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Remote ischaemic preconditioning suppresses endogenous plasma nitrite during ischaemia-reperfusion: a randomised controlled crossover pilot study

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Abstract

Aim: To test the hypothesis that remote ischaemic preconditioning (RIPC) increases circulating endogenous local and systemic plasma [nitrite] during RIPC and ischaemia-reperfusion (IR) as a potential protective mechanism against ischaemia-reperfusion injury (IRI)**.**

Methods: Six healthy male volunteers (mean age 29.5±7.6 years) were randomised in a cross-over study to initially receive either RIPC (4 x 5 min cycles) to the left arm, or no RIPC (Control), both followed by an ischaemia-reperfusion (IR) sequence (20 min cuff inflation to 200 mmHg, 20 min reperfusion) to the right arm. The volunteers returned at least 7 days later for the alternate intervention. The primary outcome was the effect of RIPC versus control on local and systemic plasma [nitrite].

Results: RIPC did not significantly change plasma [nitrite] in either the left or the right arm during the RIPC sequence. However, compared to control, RIPC decreased plasma [nitrite] during the subsequent IR sequence by ~26% (from 118±9 to 87±5 nmol/L) locally in the left arm (P=0.008) overall, with an independent effect of -58.70 nmol/L (95% Confidence Intervals -116.1 to -1.33) at 15 min reperfusion, and by ~24% (from 109±9 to 83±7 nmol/L) systemically in the right arm (P=0.03).

Conclusions: RIPC had no effect on plasma [nitrite] during the RIPC sequence, but instead decreased plasma [nitrite] by ~25% during IR. This would likely counteract the protective mechanisms of RIPC, and contribute to RIPC's lack of efficacy, as observed in recent clinical trials. A combined approach of RIPC with nitrite administration may be required.

What is already known about this subject:

- Early studies suggested that remote ischaemic preconditioning (RIPC) was effective in preventing ischaemia-reperfusion injury (IRI)
- We and others had previously demonstrated that exogenously-applied inorganic nitrite protects against IRI
- Whether RIPC-induced elevations in endogenous nitrite provide a mechanism for RIPC remains to be established

What this study adds:

- Contrary to expectation, and other recent findings, our controlled crossover study suggests that RIPC suppresses plasma [nitrite] by ~25% during IR
- This may counteract other protective RIPC mechanisms, explaining RIPC's lack of efficacy in recent larger clinical trials
- Thus, combining RIPC with exogenously-applied nitrite may be required

Introduction

Inorganic nitrite is now recognised as a valuable reservoir of nitric oxide (NO) that is preferentially generated under hypoxic and ischaemic conditions [1-3]. We and others have demonstrated the protective effects of nitrite in ischaemia-reperfusion injury (IRI) in a variety of organs, including the heart, liver, kidney and brain [2, 4, 5]. In translational studies, we have demonstrated that beetroot juice - a rich source of dietary nitrate, which enhances circulating nitrite levels, exerted a protective effect against transient endothelial dysfunction in a forearm model of IRI [6]. And, in patients with acute myocardial infarction undergoing primary percutaneous coronary intervention, intra-coronary nitrite reduced infarct size and

major adverse cardiac events in patients with absent or only faint coronary flow [7]. However, no effect was seen with intravenous nitrite [8].

Ischaemic preconditioning (IPC) exposes a given organ to controlled ischaemic insults, which then confers protection against a subsequent prolonged ischaemic injury [9]. IPC has been shown to confer benefit to a range of organs [9-13]. Organs distant to the site of IPC may also be afforded protection remotely, through the phenomenon of remote IPC (RIPC). A typical RIPC model involves the effects of a forearm IPC stimulus on a subsequent ischaemic injury to the heart [14].

Elucidating the mechanisms of RIPC and anaesthetic preconditioning would have significant implications for a range of clinical scenarios, including cardiac anaesthesia, cardiac surgery, liver resection, percutaneous coronary intervention and thrombolysis for stroke. Thus, numerous studies have attempted to identify how RIPC confers protection to distant vascular beds. Evidence for a neurogenic mechanism of RIPC signal mediation includes the observation that autonomic antagonists such as hexamethonium and ganglion blockers inhibit protective effects of RIPC in animal and human models [15, 16]. The possibility of a humoral factor is supported by observations that serum transferred from an animal exposed to RIPC confers protection to an RIPC-naïve recipient [17, 18]. Postulated messengers include a chemokine known as stromal cell-derived factor-1 alpha [19]. It is of course possible that both humoral and neurogenic signalling is involved, as suggested by the inhibition of RIPC's protective effects following either occlusion of the femoral vein or resection of the femoral and sciatic nerves [20].

NO is one of several mediators implicated in IPC and RIPC [21, 22]. NO is produced by endothelial nitric oxide synthase (eNOS) in response to shear stress during IPC [23, 24]. NO is rapidly oxidised to nitrite, and this appears to result in a local increase in plasma [nitrite] [25]. Since nitrite is readily transported in the circulation as a source of NO,

particularly under ischaemic conditions, it was proposed to be a potential mediator of RIPC by Rassaf and colleagues in 2014 [25] [25-28]. We aimed to test the hypothesis that local and circulating plasma [nitrite] increases following RIPC in a controlled crossover forearm model of IRI.

Methods

Study population

Healthy male volunteers were recruited through the use of a circular email sent to King's College London staff and students. Exclusion criteria included the presence of any pre-existing medical conditions or treatment. Subjects fasted for 12 hours prior to each study visit. The current study was a pilot sub-study; the main study was published by Tilling et al., in 2014 [29]. The sub-study was approved by the St Thomas' Hospital Research Ethics Committee, REC No: 06/Q0702/148, and written informed consent was obtained from all participants.

Study protocol

A prospective crossover study was performed, in which subjects were randomised to initially receive either RIPC or control (no RIPC), both followed by IRI. They returned for the second study visit to receive the other intervention, at an interval of at least 7 days to avoid any carryover effect of preconditioning. All studies were conducted with the subject in a supine position in a quiet, temperature-controlled (~23^0C) room. A schematic representation of the protocol is shown in **Figure 1**.

RIPC. Following a baseline flow mediated dilatation (FMD) study (see Supporting Information), four RIPC cycles were performed, each by inflating a blood pressure cuff (Hokanson) in the left arm to 200 mmHg for 5 minutes, followed by 5 minutes deflation. Next, IRI was simulated in the right arm by inflating a blood pressure cuff in the contralateral

(right) arm to 200 mmHg for 20 minutes. FMD was repeated 20 minutes post-cuff release. Blood samples were drawn for plasma [nitrite] in both arms at baseline, immediately after each RIPC cycle, at 15 min into the cuff inflation period (ischaemia), and at 1 and 15 min after cuff deflation (reperfusion).

Control. This was similar to RIPC, except the RIPC sequence was omitted.

Measurement of plasma nitrite

Blood samples were drawn from the ipsilateral and contralateral forearm veins through an 18 gauge cannula at baseline, and then immediately after each RIPC cycle from the left and right arms, after 15 min ischaemia, and after 1 and 15 min reperfusion (see Figure 1). They were transferred to pre-chilled Lithium Heparin tubes (Vacuette) and immediately centrifuged at 4 ^{0}C for 5 minutes at 4000 g, (Mikro 220R centrifuge, Hettich GR). The haemolysis-free supernatant plasma was aspirated, rapidly frozen in Eppendorf tubes using liquid nitrogen, and stored at -80 ^{0}C until the day of analysis. The quantitative analysis of nitrite was performed using a 208i Nitric Oxide Analyser (Sievers Instruments, GE analytic instruments), as we have described previously [30]. The mean value of each duplicate measurement was taken as the final result.

Data and Statistical Analyses

All data are expressed as mean ± standard error (SEM), unless otherwise stated, except subject characteristics, which are expressed as mean ± standard deviation (SD). Data were analysed using GraphPad Prism 5. Data were compared with ANOVA. Values of $p<0.05$ were considered statistically significant.

Drug/molecular target nomenclature conform to BJP's Concise Guide to PHARMACOLOGY 2015/16 [31].

Results

Six young healthy volunteers without cardiovascular risk factors took part in the study, see **Table 1** for demographic details. The blood pressure and clinical laboratory parameters were normal. Data from all six volunteers was available for FMD measurement; however, results of the nitrite assay were only available for 5 of the 6 volunteers, due to a technical problem.

Plasma [nitrite] during RIPC cycles

The baseline plasma [nitrite] was 94.1±2.4 nmol/L. It was expected as per the hypothesis that repeated brief ischaemic episodes would elevate circulating [nitrite]. Whilst there was a numerical increase in local plasma [nitrite] in the left arm by ~11 nmol/L to 104.8±4.6 nmol/L after the first RIPC cycle, there were no statistically significant changes over the RIPC sequence, P=0.37 (**Figure 2A**). Similarly, there were no significant changes in plasma [nitrite] in the contralateral (right) arm, P=0.45 **(Figure 2B)**.

Plasma [nitrite] during IR sequence

RIPC lowered plasma [nitrite] locally in the left arm by ~26% (from 118±9 to 87±5 nmol/L; P=0.008) relative to control during the IR sequence, with an independent effect of -58.70 nmol/L (95% Confidence Intervals -116.1 to -1.33) at 15 min reperfusion, see Figure 3. This analysis includes the baseline values, which were well-matched. Excluding the baseline values, the decrease during the IR sequence itself was ~33%. RIPC also produced a systemic effect, lowering plasma [nitrite] in the right arm by ~24% (from 109±9 to 83±7 nmol/L; P=0.03). Again, this analysis includes the baseline values. Excluding the baseline values, the decrease during the IR sequence itself was ~31%.

FMD (see Supporting Information)

Discussion

RIPC did not increase plasma [nitrite] during the RIPC cycles, either in the left (ipsilateral) or right (contralateral) arm. This is in contrast to the findings of Rassaf et al. [25]. Moreover, RIPC significantly decreased local and systemic plasma [nitrite] relative to Control during the IR sequence. This suggests that the RIPC signal to decrease nitrite, which is likely to be the direct local consumption of nitrite, was transmitted systemically, resulting in decreased plasma [nitrite] in the contralateral arm. Such an effect of decreasing nitrite during IR would likely oppose the other protective mechanisms of RIPC and may contribute to the limited efficacy of RIPC in clinical trials in patients.

Whilst there was an initial numerical rise in plasma [nitrite] in the left (ipsilateral) arm of ~11 nmol/L following the first cycle of RIPC in response to the local ischaemic stimulus, this effect was not significant and not sustained with the repeated RIPC cycles and did not change systemic plasma [nitrite], as measured in the right (contralateral) arm. These results differ from those of Rassaf et al, who observed an increase in plasma [nitrite] of ~11 nmol/L (basal versus RIPC: 18±8 versus 29±5 nmol/L; $P<0.05$) in the contralateral arm following 4 cycles of RIPC compared to baseline in a similar number of healthy volunteers (n=6) as our study. They found no change in a further 6 volunteers not exposed to RIPC; however, RIPC was not directly compared to control [25]. In a separate experiment, used for transfer to the mouse heart, Rassaf et al., found that the plasma [nitrite] increased to 162±58 nmol/L in the ipsilateral arm following a single cycle. The reason for the different findings between the studies is not clear. The RIPC protocols were similar between the studies. However, the baseline plasma [nitrite] was much lower in Rassaf et al.'s study [25], than ours (18±8 nmol/L vs. 94.1±2.4 nmol/L, respectively), and indeed compared to most of the previous studies by Rassaf and colleagues, which have reported a higher range - similar to ours (e.g. 200±2 nmol/L [32], 305± 23 nmol/L [33], 351±13 nmol/L [28], 68 ± 5 nmol/L [34], 82 ± 10 nmol/L [35]). In one of these studies, Rassaf et al. demonstrated that reactive hyperaemia of

the forearm (for 5 min) increased local plasma [nitrite] from 68 ± 5 to 126 ± 13 nmol/L ($p < 0.01$) - in healthy individuals. However, in patients with endothelial dysfunction there was a numerical decrease (116 ± 12 to 104 ± 10 nmol/L; n.s.) [34]. Therefore, on the basis of Rassaf's et al. own data, elevation of endogenous plasma nitrite would be a less likely mechanism for RIPC in the patients studied in the trials (described below), the majority of whom would be expected to exhibit marked endothelial dysfunction; this may explain the limited efficacy of RIPC in such patients.

Analogous to endothelial dysfunction in disease, with associated decreases in plasma nitrite with reactive hyperaemia, we speculate that transient endothelial stunning may contribute to the mechanism that we have observed in our study. Following an initial non-significant elevation in plasma [nitrite] after the first RIPC cycle in the local arm, repeated, albeit brief cycles of RIPC/IRI, result in endothelial stunning with decreased NOS-derived NO production, and thus diminished generation of nitrite from subsequent oxidation of NO. This is coupled with an exaggerated consumption of nitrite via reduction to nitric oxide at the site of repeated short preconditioning episodes of ischaemia, constituting a local 'sink' for nitrite, that will remove circulating nitrite, resulting in a systemic deficiency. Production of reactive oxygen species on reperfusion will also scavenge NO, generating a range of nitrogen oxides with local and potentially systemic effects. Plasma [nitrite] may initially be maintained by redistribution of nitrite from the vessel wall, where it is stored, to the circulation. However, this systemic deficiency of nitrite becomes manifest through a decrease in circulating [nitrite] during the subsequent IR sequence, with a restricted capacity to elevate nitrite. Other systemic spill-over effects from the left arm subjected to RIPC, whether through the release of other chemical or neurogenic mediators, may modify the systemic endothelium's capacity to generate NO/nitrite, particularly in the zone (right arm) submitted to IRI.

Thus, the recent large-scale clinical trials of RIPC have yielded mixed results. For example, the REPAIR trial (REmote preconditioning for Protection Against Ischaemia-Reperfusion in renal transplantation), by MacAllister and colleagues, 2015, showed only a weak, non-significant effect of RIPC on the primary outcome measure of eGFR in recipients of renal transplant [36]. In the context of myocardial protection, three randomised controlled trials found a decrease in troponin (a marker of myocardial injury) following RIPC in patients undergoing coronary artery bypass graft surgery [37-39], but a larger trial showed no difference [40]. Similar debate exists in the setting of percutaneous coronary intervention (PCI), with one study observing a beneficial effect [41], but another finding that RIPC actually aggravated myocardial release of troponin and hsCRP [42]. Whether reductions in cardiac biomarkers translate into improved clinical outcomes is also unclear, and has not been demonstrated in a meta-analysis [43]. Furthermore, two recent large-scale outcome studies, using a similar protocol as used here of four 5 min upper limb RIPC cycles, in patients undergoing heart surgery (RIPHeart and ERICCA) published jointly in 2015, did not find any benefit [44, 45].

To our knowledge, the only other study, besides that of Rassaf et al. [25], and our own, that has investigated nitrite during RIPC is the very recently published study by Lambert et al. [46]. However, this parallel-group study was focussed on the effects of RIPC on ischaemia-induced sympathetic activation versus control. It also reported that IR decreased combined plasma [nitrate] and [nitrite] during reperfusion from 79±10 to 71±8 μM, whilst no decrease occurred in volunteers who had received RIPC 62±3 vs 62±2 μM. However, it should be noted that these values are ~1,000-fold higher than those of Rassaf et al. and ours, as they largely reflect plasma [nitrate], with only a minor contribution from plasma [nitrite], and are in the micromolar range measured by the Griess reaction, rather than [nitrite] measured in the nanomolar range using high sensitivity ozone chemiluminescence. Plasma [nitrite] is a much more sensitive marker of changes in NO bioavailability than [nitrate] [47].

A major limitation of this study is that it was a pilot study with a small sample size and no *a priori* power calculation was performed. Therefore, we determined our achieved power in detecting our observed effect size of 2.2 (i.e. difference in plasma [nitrite] of 31 nmol/L with mean SD 14 nmol/L during IR), with alpha 0.05, which was 98% using ANOVA. In order to detect the same effect in a prospective study with alpha 0.05, and power of 80%, we would require a sample size of 6, with an achieved power of 99.8%. The power calculations were carried out using G*Power 3.1.9.2 [48]. Therefore, whilst this was an exploratory pilot study, a study of similar size would have adequate power to detect a similar effect. This pilot study was performed in 2012, and whilst initially considered to be a negative study, we now think our findings are relevant given the positive findings reported by Rassaf et al. in 2014 [25], but moreover the failure of the recently published large clinical trials in 2015, such as REPAIR [36], RIPHeart and ERICCA [44, 45] to demonstrate a consistent protective effect of RIPC, we considered that it was now important to report our findings as they may help to explain a mechanism behind the lack of efficacy in the trials. Our crossover study unexpectedly demonstrated a relative nitrite deficit. We therefore suggest that an approach with combined RIPC and supplemented nitrite might be more effective. We had a similar sample size to that of Rassaf et al. [25], which reported an increase in [nitrite] with RIPC. However, they had a parallel group design, with n=6 for RIPC, within which the statistical comparison was made, though this does not appear to have been informed by an *a priori* power/sample size calculation, and their control group (n=6) was analysed separately.

A further limitation is that our study was not powered to detect an effect of IRI, or its prevention via RIPC using FMD, as assessment of endothelial function was not a primary aim of this pilot study, and indeed no significant effect was demonstrated. However, appropriately designed studies focussing on endothelial function have observed a protective effect of RIPC on forearm microvascular [14] and conduit artery [16] endothelial function in the contralateral arm. Whilst the study of RIPC in animal models carries the advantage of

quantifying tangible organ damage, the results must be interpreted with caution in human studies, particularly in the forearm vascular bed, which is relatively more resistant to ischaemia than other circulations.

We did, however, observe that changes in FMD were closely correlated with the absolute change in plasma [nitrite] sampled from the same arm at corresponding time periods, independent of the presence of preconditioning stimuli. Previous work has also documented the relationship between FMD and plasma levels of nitrite/nitrate[49]. As FMD is a marker of eNOS activity [50], it is therefore concluded that eNOS activity and plasma nitrite levels are linked, and this is consistent with the observation that the main endogenous source of nitrite is from oxidation of eNOS-derived NO [28].

Conclusion

In this randomised controlled study, RIPC suppressed plasma [nitrite] compared to Control during an IR sequence in both the left arm receiving the RIPC stimulus, and the contralateral right arm. Whilst this effect remains to be substantiated, it may help to explain, at least in part, the lack of efficacy of RIPC in the trials and we propose that combining RIPC with supplemented nitrite would be a more effective approach.

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Table 1 **Baseline participant characteristics**

Characteristic	Mean±SD
Age (years)	29.5±7.6
BMI (kg/m^2)	23.8±4.0
SBP (mmHg)	133±11
DBP (mmHg)	71.8±8.5
MAP (mmHg)	87.2±9.0
Hb (g/L)	146±10
Cholesterol (mmol/L)	4.70±0.90

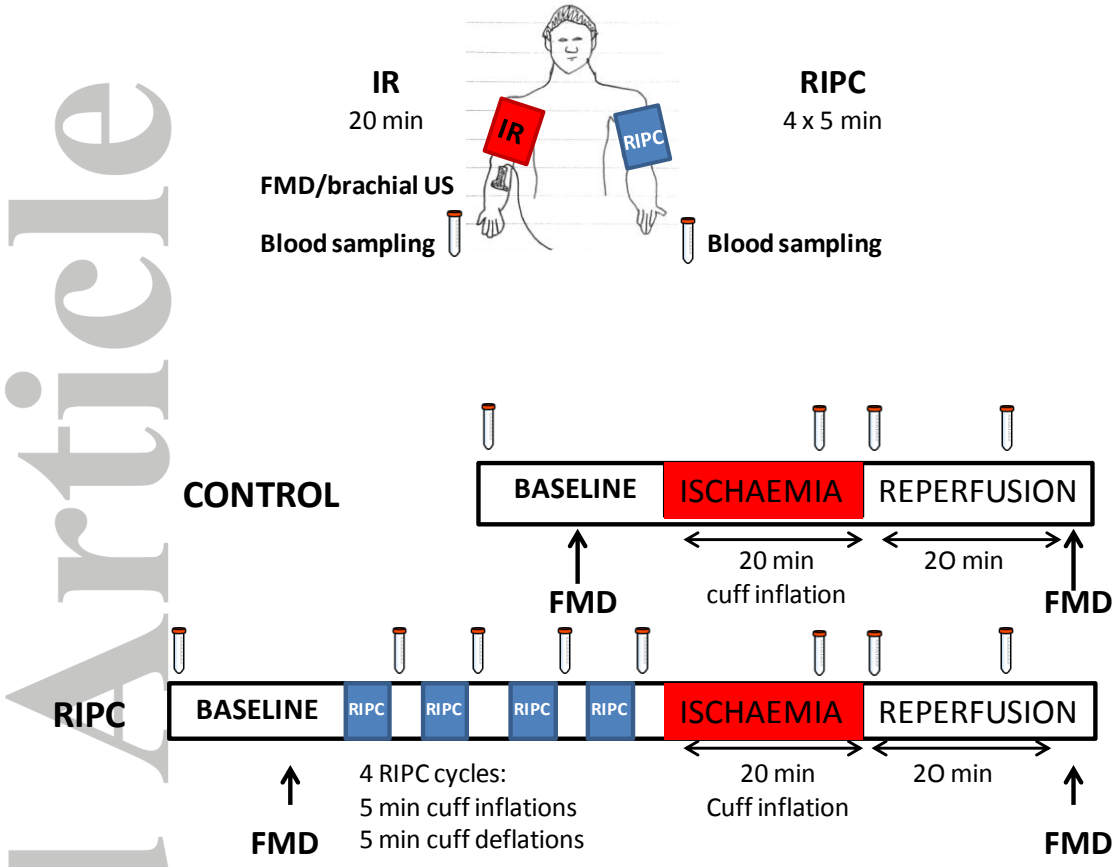

Figure 1. Schematic representation of the study protocol.

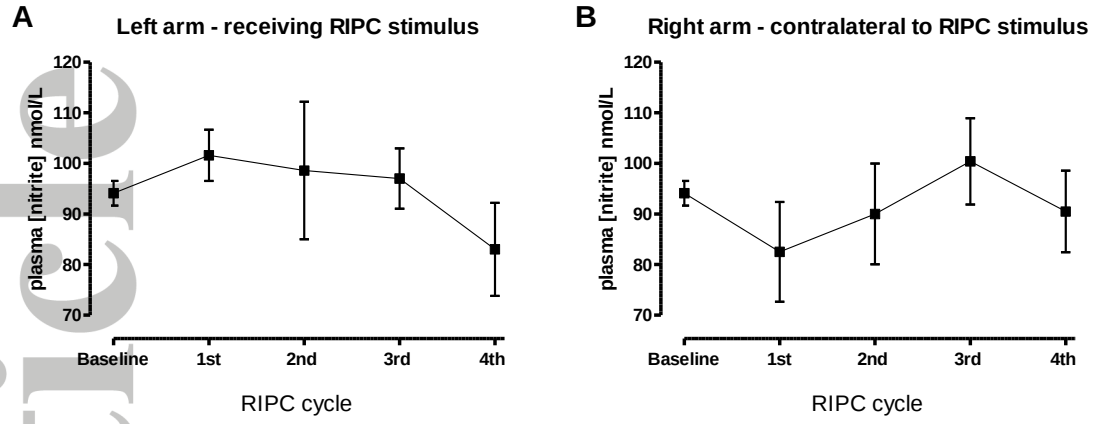

Figure 2. Effect of each Remote Ischaemic Preconditioning (RIPC) cycle performed in the left arm on plasma [nitrite] in (A) the left arm and (B) the right arm. Data expressed as mean±SEM.

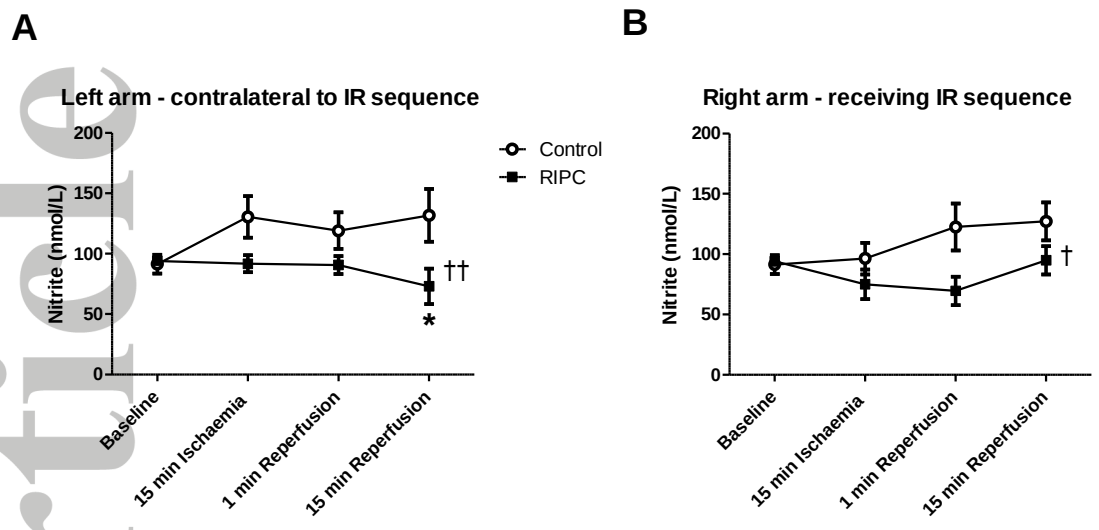

Figure 3. Effect of Remote Ischaemic Preconditioning (RIPC) performed in the left arm on subsequent plasma [nitrite] in a) the left arm and b) the right arm during an Ischaemic Reperfusion Injury (IRI) sequence applied to the right arm. Data expressed as mean±SEM. Significance shown as: †P<0.05, ††P<0.01 on ANOVA, followed by *P<0.05, Bonferroni post test of RIPC vs Control.